Insulin receptor functionally enhances multistage tumor progression and conveys intrinsic resistance to IGF-1R targeted therapy

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The type 1 insulin-like growth factor receptor (IGF-1R) tyrosine kinase is an important mediator of the protumorigenic effects of IGF-I/II, and inhibitors of IGF-1R signaling are currently being tested in clinical cancer trials aiming to assess the utility of this receptor as a therapeutic target. Despite mounting evidence that the highly homologous insulin receptor (IR) can also convey protumorigenic signals, its direct role in cancer progression has not been genetically defined in vivo, and it remains unclear whether such a role for IR signaling could compromise the efficacy of selective IGF-1R targeting strategies. A transgenic mouse model of pancreatic neuroendocrine carcinogenesis engages the IGF signaling pathway, as revealed by its dependence on IGF-II and by accelerated malignant progression upon IGF-1R overexpression. Surprisingly, preclinical trials with an inhibitory monoclonal antibody to IGF-1R did not significantly impact tumor growth, prompting us to investigate the involvement of IR. The levels of IR were found to be significantly up-regulated during multistep progression from hyperplastic lesions to islet tumors. Its functional involvement was revealed by genetic disruption of the IR gene in the oncogene-expressing pancreatic β cells, which resulted in reduced tumor burden accompanied by increased apoptosis. Notably, the IR knockout tumors now exhibited sensitivity to anti–IGF-1R therapy; similarly, high IR to IGF-1R ratios demonstrably conveyed resistance to IGF-1R inhibition in human breast cancer cells. The results predict that elevated IR signaling before and after treatment will respectively manifest intrinsic and adaptive resistance to anti–IGF-1R therapies.

multistage tumorigenesis | pancreatic neuroendocrine tumor | insulin receptor isoform A

Up-regulation of insulin-like growth factor (IGF) signaling, via alterations in the expression levels of the ligands, receptors, and/or six regulatory binding proteins, has been documented in various malignancies; both in vitro and in vivo studies have implicated this signaling axis in tumor initiation and progression (1–3). Most of the biological actions of the IGFs are mediated by the IGF-1R and insulin receptor (IR) tyrosine kinases, which share high homology at the amino acid level, particularly in the catalytic domains, differing primarily in their ligand binding specificities (4). Whereas IGF-I and insulin are highly selective for binding to IGF-1R or IR, respectively, IGF-II binds both the IGF-1R as well as an alternative splice variant of the IR, termed IR-A (5). Because of the high homology between the IGF-1R and IR, these receptors can also heterodimerize to form hybrid receptors, composed of one IGF-1R α and β subunit complex and one IR αβ complex (6). These hybrid receptors differ in their ligand binding specificities as a function of the IR isoform involved (7). Interestingly, expression of IR-A, often referred to as the fetal isoform of the IR, predominates in fetal tissues compared with their adult counterparts and has been shown to be elevated in several human malignancies (e.g., breast, colon, and lung cancer) (8).

The involvement of the IGF system in cancer is supported by experimental evidence from a number of studies that have investigated the roles of the IGF-1R in cell transformation mediated by various potent oncogenes, as well as in cell survival, proliferation, invasion, and metastasis (9, 10). These studies, combined with epidemiologic data linking alterations in the levels of circulating IGFs with cancer risk/prognosis, have focused attention on IGF-1R as a therapeutic target in cancer (11, 12). To date, most therapeutic strategies have aimed to specifically inhibit IGF-1R while sparing IR, on the basis of the concern that cotargeting IR would lead to unacceptable toxicity as a result of its role in physiologic metabolism. Because of the potential role of IR in contributing to IGF signaling, it remains unclear whether avoiding inhibition of IR could compromise efficacy. A number of companies have developed IGF-1R inhibitors (either small-molecule inhibitors or monoclonal antibodies), some of which have entered into phase III clinical trials for treating human cancer. The monoclonal antibodies typically block ligand binding with consequent down-regulation of cell-surface IGF-1R, while avoiding cross-reactivity with IR. In phase I trials, these antibodies seem to be well tolerated, with mild hyperglycemia as the most common toxicity (12).

Previous studies in our laboratory and others have demonstrated the potential utility of genetically engineered mouse models of cancer for preclinical testing of cancer therapeutic agents (13, 14). In particular, studies using the RIP1-Tag2 transgenic mouse model of cancer, in which expression of SV40 T antigens under the control of the rat insulin gene promoter (RIP) invokes a multistage pathway to pancreatic neuroendocrine tumors (PNET) of the islet β cells, have yielded useful insights regarding the success of antiangiogenic agents (15–17). In addition to oncogene expression in the ≈400 pancreatic islets in the mouse, engagement of IGF signaling components has been identified as a secondary event driving the stochastic initiation and progression of PNET tumorigenesis in this model. IGF-II expression was found to be focally activated concomitant with the initiation of β cell hyperproliferation and implicated as

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Conflict of interest statement: D.L.L. is an employee of ImClone. However, this study does not promote the ImClone drug A12.

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an antiapoptotic survival factor important for malignant tumor development via IGF-II gene knockout crosses (18, 19). The potential involvement of the IGF-1R as the signaling receptor for IGF-II was suggested by subsequent studies in which forced up-regulation of IGF-1R levels in the β cells of RIP1-Tag2 mice dramatically accelerated tumorigenesis, with the resulting tumors exhibiting a more malignant phenotype, characterized by widespread invasion and an increased frequency of lymph node metastasis (20).

Despite the striking dependence on IGF-II signaling for tumor development in this PNET model, selective therapeutic targeting of the IGF-1R using an inhibitory monoclonal antibody, currently in phase II clinical testing for a wide range of human malignancies, did not markedly impact tumor growth. We determined that the IR was serving as a second signaling receptor for IGF-II and used genetic knockout studies to confirm a role for IR in PNET tumor progression and in contributing to resistance to anti–IGF-1R therapy in vivo. The later finding was extended to human breast cancer cells, a cancer in which anti–IGF-1R targeted drugs are currently in clinical testing.

Results

Inhibitory Monoclonal Antibody to the IGF-1R Fails to Impair Tumor Growth in RIP1-Tag2 Mice. To assess the necessity of IGF-1R as a mediator of IGF-II survival signaling and as an effective therapeutic target in the RIP1-Tag2 PNET model, mice were treated with an inhibitory monoclonal antibody to the IGF-1R, IMC-A12, for a 3-week period using an intervention trial design (15) targeting growth from nascent solid tumors into lethal pancreatic adenomas and carcinomas. Immunodeficient RIP1-Tag2, $Rag^{-/-}$ mice lacking B and T lymphocytes were used to obviate concerns about neutralizing immune responses developing against the fully human therapeutic antibody; previous studies have shown that the absence of an adaptive immune system does not impact the typical multifocal and multistep PNET tumorigenesis pathway in RIP1-Tag2 mice (16). A12 has been shown to effectively block binding of IGF-I and IGF-II to the human IGF-1R [A12 comparably inhibits ligand binding to mouse IGF-1R, although it binds the mouse receptor with a slightly reduced affinity (K_D = 1.5–2.7 × 10^{-10} M)]; upon prolonged binding, A12 elicits receptor internalization and degradation, resulting in a significant reduction in growth of multiple tumor cell types (21). A12 treatment was well tolerated, with no loss of body weight or apparent morbidity throughout the 3-week trial (Fig. S1*A*). In contrast to the genetic studies demonstrating an important role for IGF-II (18), A12 treatment did not result in a significant reduction in overall tumor burden (Fig. 1*A*), although there was a 30% reduction in tumor number compared with control antibody treated mice (Fig. 1*B*; $P = 0.04$). To assess possible short-term effects of A12 on tumor cell apoptosis and proliferation, RIP1-Tag2 mice with mid- to late-stage tumors (13 to 14 weeks) were treated with A12 or control antibody, and analyzed 4 days after the first dose. A modest yet significant 1.3-fold increase in apoptotic cells was observed (Fig. 1*C*; $P = 0.01$), whereas there was no effect on tumor cell proliferation (Fig. 1*D*).

In light of the known role of the IGF-1R in invasion (22, 23) and given that RIP1-Tag2; RIP-IGF-1R double transgenic mice developed tumors with an increased invasive phenotype (20), we assessed the invasive phenotype of the islet tumors after the 3-week intervention trial with A12. A12 treated mice did not exhibit a significant alteration in the distribution of noninvasive vs. invasive carcinomas (Fig. S1*B*), although we could not rule out subtle differences in the degree of invasiveness of the resultant carcinomas in the differentially treated mice.

Owing to the modest effects of A12 on PNET tumor growth, we next assessed the biochemical effects of inhibitor treatment on IGF-1R protein levels and on inhibition of downstream signaling components. RIP1-Tag2 mice were treated with a single dose of A12 or control antibody and analyzed 6 h after injection, a procedure previously determined to markedly reduce IGF-1R and phospho-MAPK levels in a pancreatic cancer cell xenograft model (21). A12 treatment resulted in an approximately 2-fold reduction in IGF-1R protein levels, although no accompanying inhibition of AKT or MAPK kinase (MEK) phosphorylation was observed (Fig. 1*E*); on the contrary, MEK phosphorylation was curiously increased in the A12 treated tumors. Given the lack of

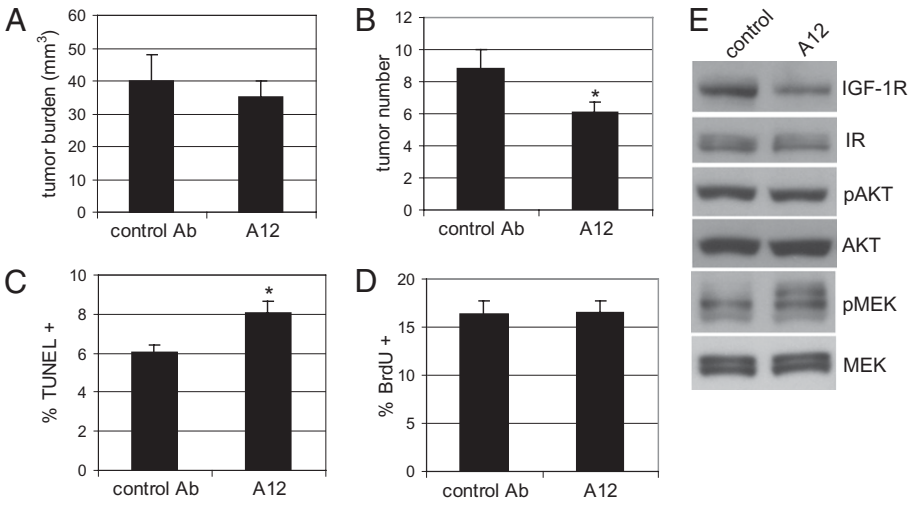

Fig. 1. An IGF-1R inhibitor does not significantly impair tumor growth in RIP1-Tag2 mice. (*A* and *B*) RIP1-Tag2; Rag-KO mice were treated in a 3-week intervention trial with the A12 anti–IGF-1R mAb or control antibody from 10 to 13 weeks of age (n = 13, control antibody treated mice; n = 15, A12 treated mice). Effects of A12 on tumor burden (*A*) and tumor number (*B*; *P = 0.04). (*C* and *D*) RIP1-Tag2 mice of tumor-bearing age (13 to 14 weeks) were treated in a short-term, 4-day trial with A12 or control antibody to assess effects on tumor cell apoptosis (*C*) as measured by TUNEL staining (*P = 0.01) and on proliferation (*D*) as measured by BrdU incorporation. Results represent values calculated from 15 to 20 tumor sections from three mice per group. Mean values plus SEM indicated; two-tailed, unpaired t test (with Welch correction for *C* and *D*) for statistical significance. (*E*) RIP1-Tag2 mice were treated with 1 mg A12 or control antibody and analyzed 6 h after injection. Pools of seven to eight tumors from three to four mice per group were analyzed by Western blotting for protein levels of IGF-1R and phosphorylated AKT (pAKT) and MEK (pMEK); blots were stripped and reprobed with antibodies to total AKT and MEK1/2 for protein load control.

inhibition of the major AKT/MAPK signaling pathways by the anti–IGF-1R antibody, the modest increase observed in apoptotic rate may be attributable to antibody-dependent cell-mediated cytotoxicity elicited by the IgG1 antibody isotype. To ensure that the inhibitory antibody was effectively entering the tumors and could bind the mouse receptor, the same protein extracts used in the previous experiment were incubated with protein A–agarose to precipitate any antibody/IGF-1R complexes. IGF-1R protein was effectively precipitated by protein A in the extracts from A12 treated mice but not control antibody treated mice (Fig. S1*C*).

Second Signaling Receptor for IGF-II Is Expressed During PNET Tumorigenesis. Because of the incongruity between the dramatic impairment in tumor growth upon genetic ablation of IGF-II and the minimal impact of the IGF-1R inhibitor, we questioned whether a second signaling receptor could be contributing to the tumor-promoting functions of IGF-II in this model. We therefore assessed expression of the other known signaling receptor for IGF-II, the fetal isoform of the IR, IR-A. The expression pattern of the two IR isoforms was also examined in different mouse organs to determine whether their expression pattern resembles that previously characterized in human tissues. As expected from the human data, expression of IR-B was found to predominate in liver and adipose, major insulin-responsive tissues, whereas IR-A expression predominated in the brain and spleen (Fig. S2), suggesting a conserved function of these splice variants. Both IR-A and IR-B isoforms were expressed in normal islets and during all lesional stages of islet tumorigenesis in RIP1-Tag2 mice, with a slight decrease in expression of IR-A relative to IR-B in tumors (Fig. 2*A*). Quantitative RT-PCR also demonstrated a modest (approximately 2-fold) decrease in total IR RNA levels in tumors compared with normal islets (Fig. S2*B*), which was in contrast to the near 7-fold increase in protein expression of the fully processed, mature form of the IR in tumors (Fig. 2*B*), indicating that this up-regulation occurs posttranscriptionally. A more modest increase in IR protein levels occurred in angiogenic islets compared with normal and hyperplastic islets, which expressed relatively low levels of mature IR protein. IGF-1R protein levels were similarly up-regulated by approximately 3-fold in angiogenic islets, with a less dramatic up-regulation in tumors compared with IR (Fig. 2*B*); as with IR, the increase in protein levels did not correlate with mRNA levels, which were decreased with progression to tumors (Fig. S2*B*). To confirm that IR expressed in islet tumor cells could serve as a signaling receptor for IGF-II, RIP1-Tag2 tumor-derived βTC4 cells that express very low levels of IGF-1R were stimulated with IGF-II. Western blotting revealed that IGF-II stimulation resulted in phosphorylation of IR in these cells (Fig. 2*C*). Hence, two known signaling receptors for IGF-II, IGF-1R and IR are expressed and up-regulated in RIP1-Tag2 tumors.

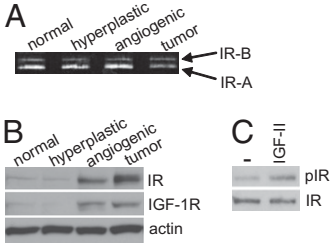

Fig. 2. The IR isoform A, a second signaling receptor for IGF-II, is expressed throughout the stages of RIP1-Tag2 tumorigenesis. (*A*) RT-PCR to detect IR isoform expression in mRNA isolated from normal islets and preneoplastic islets and tumors from RIP1-Tag2 mice. IR-A is expressed in normal islets and at all stages of RIP1-Tag2 tumorigenesis. (*B*) Expression of IR and IGF-1R protein is up-regulated in the multistage PNET tumorigenesis pathway. Protein extracts from normal, nontransgenic islets and from hyperplastic, angiogenic islets, and tumors of RIP1-Tag2 mice were immunoblotted with antibodies to the IR and IGF-1R β chain and to β-actin as a loading control. (*C*) IGF-II activates the IR in β tumor cells isolated from a RIP1-Tag2 tumor. βTC4 cells were stimulated with or without IGF-II and phosphorylated IR protein visualized after immunoprecipitation of protein extracts with an antibody to the IR β chain followed by immunoblotting with an anti-pTyr antibody.

Tissue-Specific Knockout of IR Impairs Tumor Development and Tumor Cell Survival. To investigate whether signaling via IR could contribute to tumor progression, and to generate a genetic context in which we could examine the effects of IGF-1R inhibition in the absence of IR expression, we produced RIP1-Tag2 mice containing a tissue-specific knockout of IR in their β cells (β-IRKO) using the Cre-loxP system of homologous recombination, with Cre recombinase expression driven by the RIP promoter (RIP-Cre). RIP1-Tag2; β-IRKO mice were examined at 13 weeks of age, a time point at which previous studies examining the effect of loss of IR expression in pancreatic β cells of WT mice demonstrated modest phenotypic effects (24). RIP1-Tag2; β-IRKO mice exhibited a significant 2-fold reduction in tumor volume compared with their β-IR WT littermates (Fig. 3*A*; 38 vs. 19 mm^3; $P = 0.001$). There was also a significant difference comparing RIP1-Tag2; β-IRKO mice with the heterozygous IR knockouts (31.5 vs. 19 mm^3; $P = 0.02$). The reduced tumor burden in the RIP1-Tag2; β-IRKO mice was accompanied by a significant 1.5-fold increase in the percentage of apoptotic cells compared with tumors from RIP1-Tag2; β-IRwt mice (Fig. 3*B*; $P = 0.006$), whereas there was no significant difference in tumor cell proliferation (Fig. 3*C*) or in the incidence of invasive carcinomas within the resultant tumors (Fig. S3).

To confirm that IR expression was efficiently ablated in tumors from RIP1-Tag2; β-IRKO mice, pools of tumor extracts from β-IRKO and WT tumors were examined for IR protein expression. Although the total levels of IR protein were reduced (by ≈55%) in the RIP1-Tag2; β-IRKO tumor pools compared with WT pools, a substantial amount of IR protein expression remained in the β-IRKO tumors (Fig. 3*D*, *Left*), and none of the individual tumors examined exhibited complete loss of IR expression (Fig. S4*A*). The incomplete loss of IR expression was not due to global silencing of the Cre transgene, because both IRKO and WT tumors expressed comparable levels of Cre protein, and only 1 of the 10 tumors completely lacked Cre expression. Given that β cells constitute ≈90% of the cell population of RIP1-Tag2 tumors and that normal islets (≈75% β cells) express very low levels of mature IR protein (Fig. 2*B*), it is implausible that the substantial IR expression remaining in the β-IRKO tumors comes solely from the non–β cell compartment of the tumors. Furthermore, tumor cell lines derived from four independent tumors that arose in RIP1-Tag2; β-IRKO mice all expressed WT levels of IR protein, albeit in this case with concomitant loss of Cre protein expression (Fig. S4*B*). Interestingly, examination of different stages of islet tumor progression revealed relatively efficient recombination of the IR gene in a pool of hyperplastic islet lesions, whereas progression to angiogenic lesions and tumors was associated with comparably more incomplete recombination at this locus (Fig. 3*D*, *Middle*). Collectively, the results are suggestive of a selective pressure to maintain some degree of IR signaling both in vivo and in vitro for tumor cell growth/survival, either by loss of Cre expression (as was the case in the tumor cells lines), or by an as of yet undefined epigenetic mechanism (resulting in incomplete recombination at the IR-lox locus). Notably, IGF-1R protein levels were markedly elevated in RIP1-Tag2; β-IRKO tumors (Fig. 3*D*, *Left*), potentially serving as an additional mechanism to compensate for suspected cell lethal effects of IRKO in PNET cancer cells, thereby masking the potential impact from loss of IR expression, particularly with regard to effects on tumor growth, apoptosis,

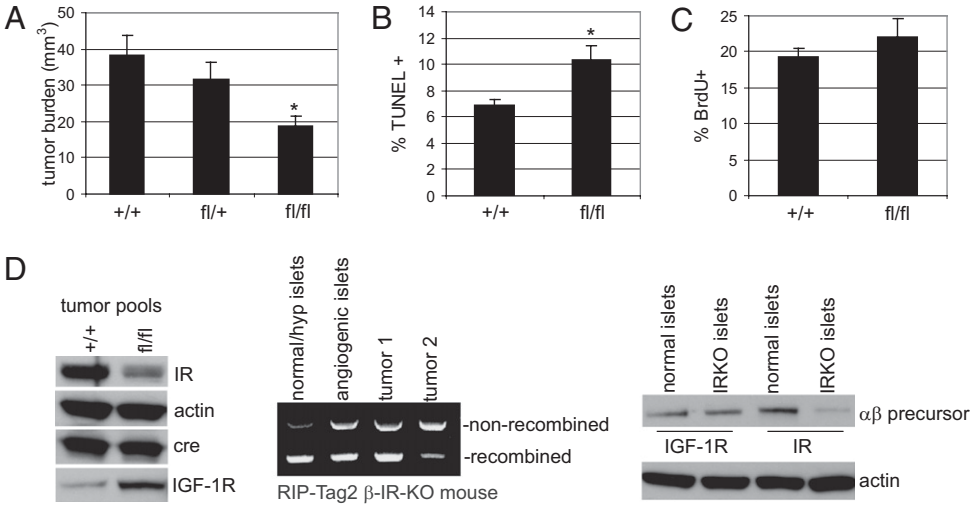

Fig. 3. Tissue-specific knockout of IR in β cells impairs PNET tumorigenesis. (*A–C*) Double transgenic RIP1-Tag2, RIP-Cre mice of the indicated IR genotypes were analyzed at 13 weeks of age: β-IR WT (+/+); heterozygous β-IRKO (fl/+); and β-IRKO (fl/fl). (*A*) Effects on tumor burden; $n = 13$ (+/+), $n = 22$ (fl/+), $n = 37$ (fl/fl); $^*P = 0.001$ compared with β-IRwt mice, $^*P = 0.02$ compared with fl/+ mice (*B* and *C*) Effects on apoptosis (*B*; $^*P = 0.006$) and proliferation (*C*; $P = 0.33$); $n = 17$ tumor sections from five mice (+/+), $n = 18$ tumor sections from eight mice (fl/fl). Bars represent mean values ± SEM. Two-tailed, unpaired *t* test (with Welch correction for *B* and *C*). (*D*) Selection against complete loss of insulin/IGF signaling receptors in tumor cells. IR and IGF-1R protein levels in pooled tumor extracts from RIP1-Tag2, β-IRKO mice (*Left*). Tumor pools consist of equal protein amounts from 5 tumors from 4 mice (IR +/+) and 10 tumors from 10 mice (IR fl/fl). Recombination of IR gene in different stages of islet tumor development in a RIP1-Tag2; β-IRKO mouse (*Center*). IR and IGF-1R protein levels in islet pools from β-IRKO mice (*Right*; αβ precursor depicted owing to low levels of processed, mature protein).

and invasion. IGF-1R protein levels were not increased in β-IRKO islets from mice that did not express the SV40 T-antigen oncogene (Fig. 3*D*, *Right*). Moreover, the islets in this context exhibited a more complete ablation of IR expression.

Loss of IR Expression Sensitizes Tumors to IGF-1R Inhibition. Having established that IR was functionally contributing to PNET tumorigenesis in RIP1-Tag2 mice, and that the IGF-II-binding isoform was expressed throughout the pathway, we considered the possibility that this parallel signaling pathway might constitute a basis for the intrinsic resistance we observed to anti–IGF-1R therapy, in terms of minimal reduction of AKT phosphorylation and minimal therapeutic efficacy. To test this hypothesis, tumor-bearing RIP1-Tag2; β-IRKO mice were treated with a single dose of A12 or control antibody and analyzed 6 h after injection, as in Fig. 1*E*. This single treatment resulted in an 80% decrease in IGF-1R protein levels, which was now accompanied by a near complete inhibition of AKT phosphorylation. The increased efficacy of A12 in inducing IGF-1R down-regulation in the setting of IR-KO is curious and could indicate enhanced

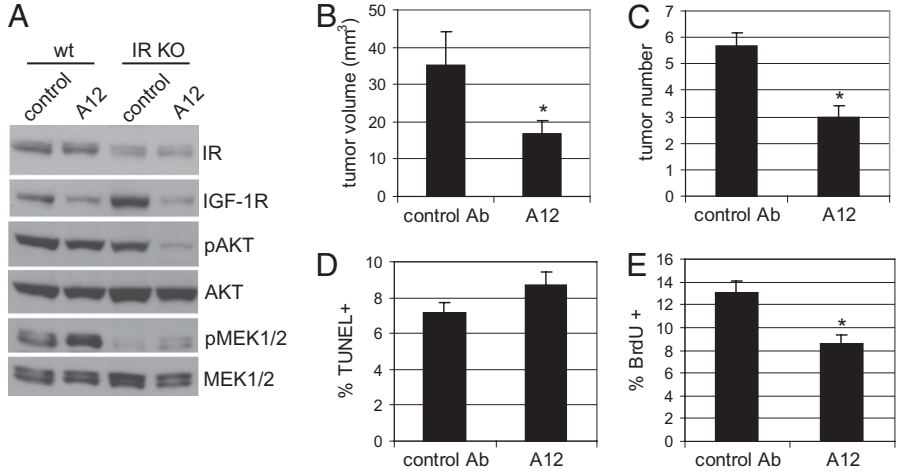

Fig. 4. Loss of IR expression sensitizes PNET tumors to IGF-1R inhibition. (*A*) RIP1-Tag2; β-IRKO mice were treated with 1 mg A12 or control antibody and analyzed 6 h after injection. Pools of four tumors from at least two mice per group were analyzed. As a comparison, protein extracts from similarly treated WT RIP1-Tag2 mice were analyzed alongside. (*B* and *C*) RIP1-Tag2; β-IRKO mice were treated in a 2-week intervention trial with A12 or control antibody from 12 to 14 weeks of age ($n = 13$ for control antibody, $n = 17$ for A12 treated mice). Effects on tumor burden (*B*; $^*P = 0.02$) and tumor number (*C*; $^*P = 0.0005$). Mann-Whitney test for statistical significance. (*D* and *E*) RIP1-Tag2; β-IRKO mice were treated with a single dose of A12 antibody and effects on tumor cell apoptosis (*D*; $P = 0.1$) as measured by TUNEL staining (≥15 tumor sections from four to seven mice per group analyzed), and proliferation (*E*; $^*P = 0.02$) as measured by BrdU incorporation (≥22 tumor sections from six to seven mice per group analyzed) were analyzed after 24 h. Mean values +SEM are indicated. Two-tailed, unpaired *t* test (with Welch correction for BrdU analysis).

function in inhibiting IGF-1R homodimers as compared with IGF-1R/IR hybrid receptors; although at least in the case of the human receptors, A12 was demonstrated to effectively block ligand binding and signaling of hybrid receptors (21). As in the WT RIP1-Tag2 mice, a slight increase in MEK phosphorylation was observed with A12 treatment, although total pMEK levels in the anti–IGF-1R antibody treated IR-KO tumors were still diminished compared with the WT tumors (Fig. 4*A*).

We next subjected the RIP1-Tag2; β-IRKO mice to a shortened 2-week intervention trial with A12, treating from 12 to 14 weeks of age. The 2-week trial was designed to minimize the potential for confounding results of a neutralizing antibody response to the fully human antibody that could in principle occur within the time frame of a typical 3-week intervention trial in the immunocompetent RIP1-Tag2; β-IRKO mice. The multiplicity of transgenic and gene-targeted alleles precluded incorporation of the homozygous Rag1-KO allele to obviate this potential, as was used in the initial assessment of A12 shown in Fig. 1. The later start time (12 weeks in the β-IRKO mice compared with 10 weeks in the IR WT mice) was chosen to achieve a comparable tumor burden at the end point of the trial in control antibody treated mice. In contrast to the negligible efficacy of A12 in RIP1-Tag2 mice with WT β cell IR levels, A12 treatment of RIP1-Tag2; β-IRKO mice resulted in a significant 2-fold decrease in tumor burden (Fig. 4*B*; $P = 0.02$) and tumor number (Fig. 4*C*; $P = 0.0005$). Inhibition of tumor cell proliferation was the likely mechanism accounting for the reduced tumor burden, in that a single dose of A12 resulted in a significant 34% reduction in the percentage of proliferating cells ($P = 0.02$), whereas the percentage of apoptotic cells was not significantly altered (Fig. 4 *D* and *E*). In light of the robust inhibition of pAKT, the lack of substantial effect on apoptosis was unexpected. It is possible that analysis at other time points might have revealed more of an effect or that other cell survival mechanisms, such as autophagy, could be affected by A12. Because apoptosis is associated with aberrant β cell proliferation in RIP1-Tag2 mice, a decrease in the rate of cell proliferation by A12 could be abating the impetus for apoptosis. It is also surprising that interference with IGF-1R by A12 treatment did not appreciably affect the invasive phenotype of the resultant tumors (Fig. S5), although we could not rule out subtle effects. Thus, in this IGF-II–driven cancer model, both IR and IGF-1R signaling must be dampened to measurably inhibit AKT activation and maximally hinder tumor growth.

Differential IGF1R/IR Ratios Correlate with Sensitivity of Human Breast Cancer Cells to IGF-1R Inhibition. To begin extending our findings to human cancer, we next investigated whether the IR might also contribute to insensitivity to IGF-1R inhibition in human breast cancer cells. Most human breast carcinomas overexpress IGF-1R and/or IR (25, 26), and numerous studies support the important role of IGF-signaling in this disease (27, 28). Clinical trials are currently underway in breast cancer with a variety of IGF-1R targeting agents, including A12. As shown in Fig. S6, A12 treatment significantly inhibited IGF signaling in cell lines with high IGF-1R to IR expression ratios (MCF-7, T47D), whereas it was comparably ineffective in those characterized by low IGF-1R to IR ratios (MDA-MB-231 and -157). Of note, MDA-MB-231 cells also displayed marked resistance to A12 in a s.c. xenograft tumor model (Fig. S7), in contrast to MCF-7 cells, which were previously demonstrated to be highly sensitive to A12 in this assay (21).

We next asked whether the responses to A12 could be enhanced by suppressing levels of IR expression. As shown in Fig. S8 *A* and *B*, knockdown of IR expression with anti-IR siRNAs markedly sensitized the more resistant MDA-MB-231 cells to A12, as evidenced by a now highly effective inhibition of IGF-1R/IR and AKT phosphorylation in response to IGF stimulation. To assess the functional effects of IR knockdown, stable knockdown of IR expression was achieved in these cells by lentiviral-mediated transduction with an IR-targeting shRNA (Fig. S8*C*), and the ability of A12 to interfere with anchorage-independent growth was assessed. Previous studies have demonstrated that growth of some cancer cells in anchorage-independent conditions is critically dependent on IGF-1R expression (10). Although A12 was able to significantly inhibit colony formation (by 45%) in MDA-MB-231 cells transduced with a nonsilencing (NS) shRNA (Fig. 5*A*; $P < 1 \times 10^{-6}$), knockdown of IR expression appreciably enhanced this effect, producing a 75% reduction in colony number compared with control treated cells ($P < 1 \times 10^{-10}$). IR knockdown in the absence of A12 treatment also modestly inhibited colony formation, by ≈30% ($P < 0.0001$). In contrast to the effects of IR knockdown and A12 treatment on anchorage-independent growth of the MDA-MB-231 cells, monolayer growth of these cells, as determined in an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, was unaffected by both IR knockdown and A12 treatment (Fig. S8*D*), consistent

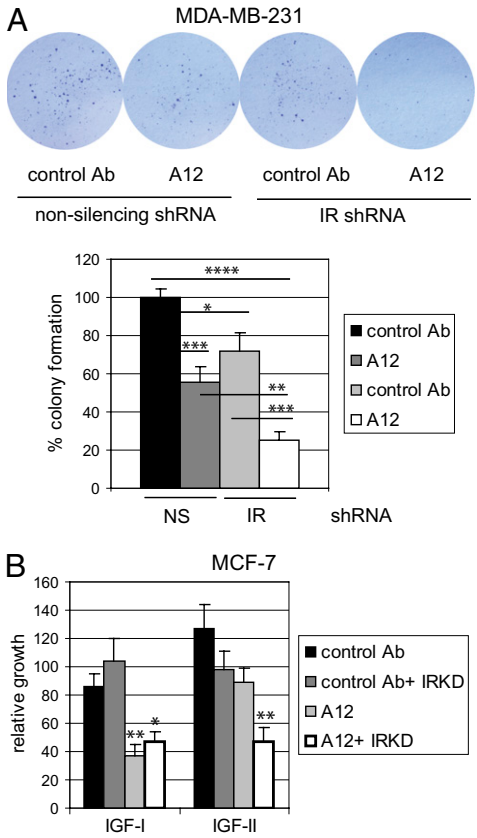

Fig. 5. Enhanced growth inhibition of breast cancer cells by IGF-1R inhibitor in the setting of IR knockdown. (*A*) MDA-MB-231 cells stably transduced with an NS or IR-targeting shRNA were grown in soft agar in the presence of control or A12 antibodies. Images (*Upper*) and values (*Lower*) from two independent experiments, repeated in triplicate are depicted; Mean values plus SD are indicated and expressed as percentage colony formation relative to that of NS-shRNA expressing, control antibody treated cells; $*P < 0.0001$, $**P < 1 \times 10^{-5}$, $***P < 1 \times 10^{-6}$, $****P < 1 \times 10^{-11}$; two-tailed, unpaired *t* test. (*B*) MCF-7 cells were transfected with control or IR siRNAs and serum-starved cells were stimulated 48 h later with 10 nM IGF-I or 20 nM IGF-II or left unstimulated in the presence of control or A12 antibodies (100 nM) for an additional 48 h. Cell growth was assessed by incubation with MTT and values normalized relative to growth of control treated, unstimulated cells. Mean values plus SEM are indicated. Results representative of four independent experiments performed in triplicate. $*P < 0.01$, $**P < 0.001$, two-tailed, unpaired *t* test. (Magnification: *A*, ×3.4.)

with previous reports demonstrating that whereas IGF-1R expression is critical for growth of cells in anchorage-independent conditions, it can be dispensable for growth and survival of certain cancer cells (including MDA-MB-231 cells) in monolayer cultures (10, 22, 29).

Because knockdown of IR appreciably enhanced the efficacy of A12 in the MDA-MB-231 cells, which have a relatively high ratio of IR to IGF-1R expression, we wondered whether the efficacy of A12 could also be enhanced by suppression of IR in the already sensitive MCF-7 cells, which have very high levels of IGF-1R, and hence a high IGF-1R to IR ratio despite expressing IR at appreciable levels. In fact, IR knockdown in MCF-7 cells effected a more complete abolition of IGF-stimulated phosphorylation of IGF-1R/IR and MEK in response to A12 (Fig. S8*B*). To test whether this further sensitization could be observed functionally, we assessed the effect of IR knockdown on the ability of A12 to inhibit MCF-7 cell proliferation. IR knockdown did not hinder basal MCF-7 cell growth or significantly affect growth in response to IGF-I or II (Fig. 5*B*). As has been previously reported (21), A12 was effective in inhibiting proliferation of MCF-7 cells in response to IGF-I, and we did not observe further benefit upon IR knockdown (Fig. 5*B*). In marked contrast, A12 was not able to significantly inhibit proliferation of MCF-7 cells in response to IGF-II ($P = 0.07$), except in the setting of IR knockdown, in which case a highly significant 63% decrease in proliferation compared with unstimulated levels was observed ($P = 0.0008$). Thus, optimal impairment of growth of high IR-expressing breast cancer cells via IGF-1R inhibition is achieved by concomitant knockdown of IR expression.

Discussion

In this study, we demonstrate that the IR is an integral component of the tumor-promoting IGF signaling axis, contributing to tumor progression and mediating therapeutic resistance to a pharmacological inhibitor of the IGF-1R in a mouse model of pancreatic neuroendocrine cancer. Compared with the IGF-1R, the IR has received relatively little attention in regard to its potential cancer promoting properties, likely reflecting recognition of its important role in maintaining glucose homeostasis with consequent concerns about toxicity upon therapeutic intervention; there is, however, mounting evidence that the IR may also contribute significantly in transducing protumorigenic effects of IGFs (30, 31), including a complementary study recently published by Zhang et al. demonstrating a role for IR in growth and metastasis of breast cancer cells in vivo (32). Several reports have documented the overexpression of IR, and in particular the IR-A isoform, in human cancers (5, 33–37). We hereby provide evidence that up-regulated IR expression can be functionally relevant to tumor progression. Levels of mature IR protein were markedly increased during multistep progression to tumors in the RIP1-Tag2 PNET model, and targeted deletion of the IR gene in pancreatic β cells of these mice impaired tumor progression. Interestingly, whereas efficient recombination of the IR gene was tolerated in normal islets (24) and preneoplastic islet hyperplasias, inefficient recombination was observed in more advanced stages, suggestive of a selective pressure to maintain IR expression for tumor development. The results implicate IR as an essential gene in the oncogene-expressing cancer cells, in contrast to normal islet β cells, hinting that tumor cells may be more dependent on IR signaling than their normal counterparts.

In addition to potentially promoting cancer via serving as a second signaling receptor for IGF-II, binding to insulin itself may also contribute to protumorigenic signaling of IR (38–42). Of relevance, epidemiological evidence has demonstrated a link between hyperinsulinemic states (such as obesity and insulin resistance) and increased cancer risk (43–46), and studies in animal models have presented evidence for a stimulatory effect of hyperinsulinemia (47, 48) and conversely, an inhibitory effect of insulin deficiency (49–51) on cancer growth. In this regard, the reduced tumor burden and increased apoptosis observed in the RIP1-Tag2; β-IRKO mice may in part be explained by a defect in insulin signaling. Interestingly, an increased responsiveness of cultured human breast cancer cells to insulin in the setting of down-regulated IGF-1R has been reported (52), thus raising the possibility that increased insulin signaling via IR, in parallel with IGF-II→IR signaling could also be contributing to resistance to anti–IGF-1R therapy in the RIP1-Tag2 PNET model.

Reflecting the expected negative consequences of inhibiting normal metabolism controlled by IR signaling, recent therapeutic strategies to target the IGF pathway have focused on the development of specific inhibitors to the IGF-1R. A current challenge in such efforts is to identify biomarkers that can predict response. It is curious that IR has not been prominently identified as a resistance factor in reports describing such attempts (53). Given that IR levels can be regulated posttranscriptionally, the widespread utilization of RNA expression levels to screen for potential biomarkers may in part explain this oversight. Although a selective IGF-1R targeting strategy may be effective in IGF-I driven cancers with high IGF-1R expression, as demonstrated in an activated Kras-driven mouse model of basal-like breast cancer (54), it may not be as effective in the setting of high IR to IGF-1R expression ratios, particularly in cancers coexpressing IGF-II and IR-A, as has been recently demonstrated in osteosarcoma (36) and herein in the RIP1-Tag2 PNET model.

We demonstrate that interference with IR expression in tumors, thereby increasing levels of IGF-1R relative to IR, was required for effective suppression of AKT activation and inhibition of tumor cell proliferation and tumor growth in a therapeutic intervention trial with the A12 IGF-1R inhibitor. Thus, inhibition of tumor growth via targeting the IGF signaling pathway in this IGF-II dependent tumor type requires concomitant suppression of both of its receptor tyrosine kinases, IGF-1R and IR. If this result is translatable, then one can predict that cotargeting IR and IGF-1R with one of the newly described IGF-1R/IR small-molecule inhibitors that are currently being tested in phase I clinical trials (55) will prove to have broader efficacy than drugs that target IGF-1R exclusively; data from future and ongoing clinical trials will address both this question as well as elucidate whether such dual targeting can be achieved with acceptable toxicity. Notably, two phase III clinical trials of another IGF-1R inhibitory monoclonal antibody (figitumumab) in combination with either chemotherapy or erlotinib (EGFR inhibitor) in non–small-cell lung cancer were recently discontinued prematurely owing to evident lack of survival benefit; it would be of interest to determine whether intrinsic resistance conveyed by preexisting high levels of IR signaling might underlay the lack of clinical benefit; if so, future trials involving preselection of patients with tumors that express low levels of IR-A and IGF-II might produce a responsive cohort with demonstrable efficacy.

In addition to the resistance mediated by IR implicated herein, additional mechanisms of intrinsic or acquired resistance to IGF-1R and potentially to IGF-1R/IR dual targeting drugs can be envisioned, including amplification/activation of other receptor tyrosine kinase signaling pathways (e.g., EGFR) and/or mutations in downstream signaling pathway components (e.g., PTEN, PI3K). In fact, increased activity of the EGFR pathway has been identified as a potential resistance mechanism (56, 57). It is of note that in addition to dependence on IGF signaling, RIP-Tag2 tumors have also recently been demonstrated to exhibit sensitivity to pharmacological or genetic inactivation of EGFR (58), raising the possibility that signaling via EGFR can also influence the efficacy of IGF pathway targeting strategies in this model. It was surprising that effective blockade of IGF signaling pathways by A12 did not appreciably affect the invasive phenotype of the resultant tumors, in contrast to the proinvasive effects observed upon IGF-1R overexpression (20), perhaps

suggesting that IGF-1R can enhance but is not obligatory for cancer cell invasion in this model. The alternative possibility remains that earlier intervention or a longer duration of therapeutic modulation of IGF-1R would prove to impact the invasive phenotype.

Similar to the mouse PNET tumors, suppression of IR expression was also required for A12 efficacy in the human breast cancer cells where IR was more abundantly expressed than IGF-1R. It is of note that combined targeting of IGF-1R and IR was remarkably effective in inhibiting anchorage-independent growth of MDA-MB-231 cells despite the fact that this cell line is atypical, in that it contains activating mutations in both RAF and RAS (59), which are infrequent in human breast cancer. The result suggests that IGF-1R/IR signaling may convey distinct signals to those transmitted by the activated Ras pathway, encouraging of the potential generality and importance of this dual signaling axis. Moreover, even MCF-7 cells, which overexpress IGF-1R but also express significant levels of IR-A, required IR knockdown for A12 to fully inhibit IGF-II mediated growth. As such, in the event of high IR-A expression in an IGF-II expressing cancer, alternative strategies of inhibiting IGF signaling may be warranted, such as cotargeting of the IR (or specifically IR-A, if possible), targeting of downstream signaling pathway components (e.g., PI3K), or targeting the ligand itself, the latter strategy already implemented clinically for inhibition of the VEGF pathway with the US Food and Drug Administration–approved drug bevacizumab. The possibility of targeting the IGF-II ligand has recently been suggested as a potential therapeutic strategy for IGF-II dependent osteosarcoma (36) and could be further explored in preclinical and clinical trials of this and other cancer types, given the recent development of inhibitory monoclonal antibodies to IGF-II (60) and a soluble IGF-II trap based on a modified IGF-IIR (61).

The dysregulation of IGF-II expression and/or its bioavailability is a common event in human cancer (1). Strikingly, IGF-II has been identified as the most highly overexpressed gene in colon cancer (62), and its increased expression has been associated with colon cancer risk (63, 64), suggesting a potential functional role in the disease pathogenesis. Evidence in breast cancer suggests that IGF-II, rather than IGF-I, may play the predominant role, with expression commonly found in tumor associated stromal cells and occasionally in the tumor cells themselves (27). Given the prevalence of IGF-II deregulation in human cancer, interfering with its signaling pathway is of considerable therapeutic interest. Our studies in the prototypical IGF-II driven RIP1-Tag2 mouse model of multistage carcinogenesis demonstrate, in vivo, a role for IR in tumor progression and, importantly, in eliciting intrinsic resistance to IGF-1R targeting therapy. It can additionally be envisioned that up-regulation of the IR signaling axis may afford mechanisms of adaptive resistance to anti-IGF-1R therapeutics in tumors that initially respond but then relapse. The potential role for IR in contributing to innate and adaptive resistance to selective IGF-1R targeting strategies encourages consideration of neoadjuvant and other trial designs in which patients are either preselected for low vs. high IR-A and IGF-II levels and/or wherein relapsing tumors can be analyzed for adaptive effects on IR-A and IGF-II expression.

Experimental Procedures

Transgenic Mice Breeding. The generation and characterization of RIP1-Tag2 transgenic mice (65), compound RIP1-Tag2, Rag1 knockout immunodeficient mice (16), RIP2-Cre transgenic mice (66, 67), and IRlox mice containing loxP sites flanking exon 4 of the IR (24, 68) have been previously described. All mice were backcrossed into the C57BL/6 background for at least 10 generations. RIP1-Tag2, RIP-Cre, and IRlox mice were intercrossed to generate RIP1-Tag2; RIP-Cre; $IR^{+/+}$, $IR^{fl/+}$, and $IR^{fl/fl}$ mice for analysis of the effect of loss of pancreatic β cell IR expression on the RIP1-Tag2 tumorigenesis pathway. Littermate controls were used in all experiments. Mouse experiments were performed in accordance with the University of California, San Francisco institutional review board guidelines.

Pharmacological Trials of IGF-1R Inhibition. The fully human monoclonal inhibitory antibody to the IGF-1R, A12 and an isotype control antibody to an irrelevant antigen (anti-KLHλ IgG1) were obtained from ImClone Systems. Mice were treated every 3 days with 1 mg A12 or control antibody by i.p. injection, as previously described (21). Immunodeficient RIP1-Tag2; Rag-KO mice were used in the 3-week intervention trial to prevent generation of neutralizing antibody responses; immunocompetent mice were used in the remaining experiments involving ≤2 weeks of treatment.

Cell Culture Experiments. ***MTT assays.*** To assess cell growth, the Vybrant MTT Cell Proliferation Assay Kit (Molecular Probes) was used according to manufacturer's instructions. Cells were plated in 96-well plates at a density of 5,000 cells per well and absorbance read at 570 nm after the indicated treatments. ***Soft agar assays.*** Cells were resuspended in 0.35% low melting temperature agarose in DMEM (containing 10% FBS) and plated at a density of 5,000 cells per well in a six-well plate over a bottom layer of 0.5% agarose. Both top and bottom agar layers contained 1 μg/mL puromycin and 100 nM control anti-KLHλ or A12 antibodies, and the agar was overlayed with media supplemented with 20 nM IGF-II to stimulate IGF-mediated growth. Colonies were allowed to develop for 2 weeks. Plates were stained with 0.005% crystal violet and colonies counted under a dissecting scope; images captured with Leica DFC500 camera.

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